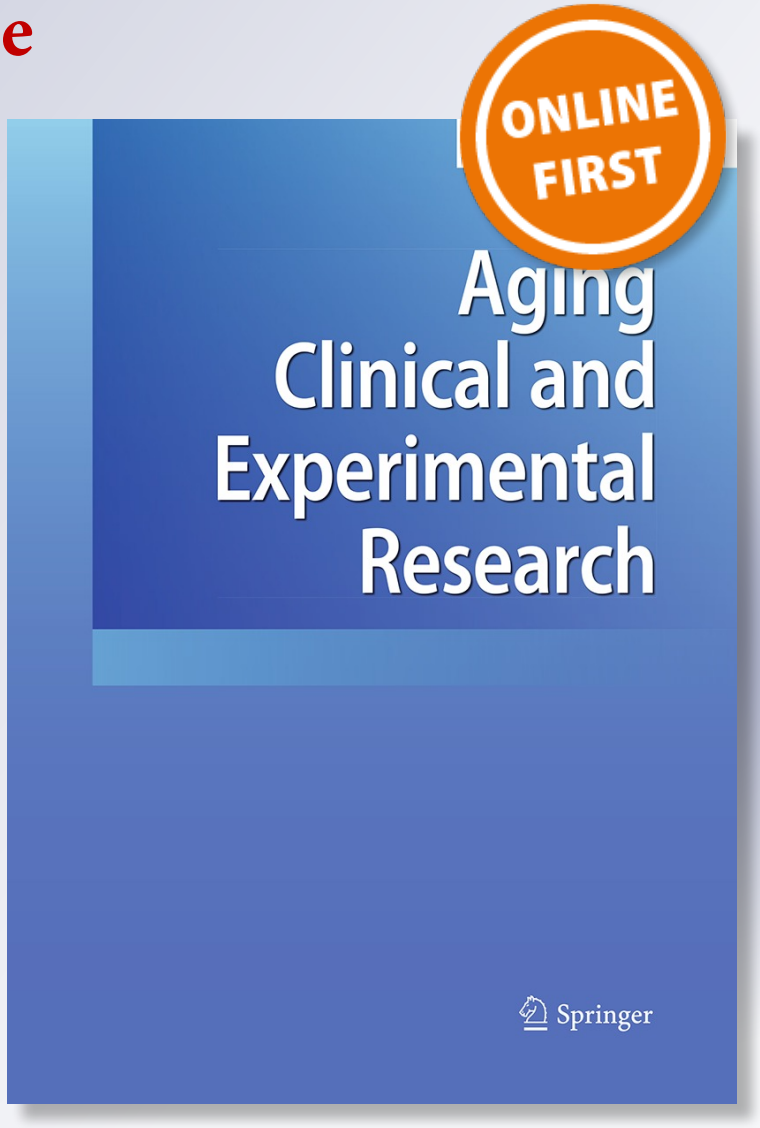
Cytoskeleton involvement in lithium-induced SH-SY5Y neuritogenesis and the role of glycogen synthase kinase 3β

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ORIGINAL ARTICLE

Cytoskeleton involvement in lithium-induced SH-SY5Y neuritogenesis and the role of glycogen synthase kinase 3β

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Abstract Lithium modulates signals impacting on the cytoskeleton, a dynamic system contributing to neural plasticity at multiple levels. In this study, SH-SY5Y human neuronal cells were cultured in the absence (C) or in presence (Li) of a 0.5 mM Li_2CO_3 (i.e. 1 mM lithium ion) for 25–50 weeks. We investigated the effect of this treatment on (1) morphological changes of cells observed using Hemalun eosin staining assay, (2) cytoskeletal changes by indirect immunofluorescence (IIF) staining of microtubules (α-tubulin) and heavy neurofilaments subunits (NF-H) and by measuring the expression rate changes of genes coding for receptor for activated C kinase (RACK1), casein kinase2 (CK2) and thymosine beta-10 using cDNA arrays technology, (3) cell adhesion properties by IIF staining of β-catenin protein. Besides, we have tried to understand the molecular mechanism of lithium action that triggers changes in cytoskeleton and neurites outgrowth. Thus, we examined the effect of this treatment on glycogen synthase kinase 3 (GSK3) expression and activity using western blotting of GSK3 and phosphorylated β-catenin, a downstream GSK3 target protein. Our results showed that lithium treatment reduces axon length, increases axonal spreading, enhances neurites growth and neurites branching with an increase of growth cone size. Moreover, genes coding for CK2 and thymosine beta-10 were significantly up-regulated, however, that coding for RACK1 was down-regulated. The most interesting result in this work is that mechanism underlying lithium action was not related to the inhibition of GSK3 activity. In fact, neither expression rate nor activity of this protein was changed.

Keywords Lithium · Cytoskeleton · Neuritogenesis · GSK3

Introduction

Aging, at the cellular level, is associated with certain aspects of degeneration, including a degeneration of cells and tissues similar to that which can be found in a broad range of age-associated diseases, such as cancer, cardiovascular failure, and neurodegenerative disorders such as Alzheimer's disease (AD) which constitutes the most common type of disturbance in the human aging brain [1–3]. In this disease, the brains of patients exhibit at least three signs of neurodegeneration: dystrophic neurons, intraneuronal neurofibrillary tangles (NFT), and extracellular amyloid deposits within cerebral vessel walls and neuritic plaques [4, 5].

Accumulating studies have suggested that lithium is a neuroprotective agent against diverse insults. Indeed, increasing evidence suggests that lithium can be used in the treatment of acute brain injuries (e.g., ischemia) and chronic neurodegenerative diseases (e.g., Alzheimer's disease) [6]. Lithium is also known to be neurotrophic and is reported to enhance neurites extensions and synaptogenesis [7]. In this context, we recently reported that chronic treatment by therapeutic relevant lithium concentrations could favor neuroprotective and neurotrophic effects in mice brains. These effects were proved, in part,

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by the stimulation of neurogenesis and neurites growth in the hippocampus and enthorinal cortex [6]. In vitro, Italia et al. [8] showed that neurite extensions were significantly increased in SK-NMC cells treated with lithium (2.5 mM) for 24 h, this suggests lithium-induced neuritogenesis [8].

Despite its wide use in treatment of several brain diseases, the mechanisms underlying the lithium therapeutic action remain unclear [9–12]. However, multiple direct and indirect molecular targets of lithium have been identified over the years [13–16]. The most important function of lithium is to inhibit glycogen synthase kinase 3 (GSK3) [17]. In addition to targeting GSK3, lithium also affects the activity of other protein kinases/phosphatases, such as PKA and PP2A [18–20]. Lithium can directly or indirectly modulate proteins involved in neuronal survival/differentiation independent of GSK3. For example, lithium was shown to promote the action of anti-apoptotic proteins, such as HSP, BDNF, IGF, VEGF, PKC, ERK, CREB, cAMP, Bcl-2, GRP78 and PI3 K/AKT. It inhibits pro-apoptotic signaling such as the expression/activation of p53, Bax and tau [21–24].

In this study, we used SH-SY5Y cells to analyse the effect of long-term lithium treatment, at therapeutic concentration (0.5 mM), on neuritogenesis. Cells were treated for more than 10 months and the effect of this treatment on cytoskeletal changes, neurites growth, and cells adhesion was analysed. Besides, the process by which signaling molecules led to these effects was investigated.

Materials and methods

Chemicals and reagents

Lithium carbonate (Li_2CO_3), pro analysis grade, was from Prolabo/Rhone-Poulenc (France). Monoclonal antibodies against phosphorylated and unphosphorylated GSK3α/β were from Cell Signaling (Victoria, BC, Canada). Anti β-catenin, anti α-tubilin clone DM1, anti-mouse IgG-FITC antibody were from Sigma-Aldrich. Anti p-NF-H (RNF405) was from Santa Cruz Biotechnology. Super-Signal® West Pico Chemiluminescent substrate was from Pierce biotechnology (USA). Atlas™ Nylon cDNA arrays (Human neurobiology Arrays, 7736-1) were from CLONTECH. Fetal calf serum (FCS) and other cell culture reagents were from Institut Jacques Boy, Reims, France. Proteases inhibitors, i.e., phenylmethanesulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), and aprotinin were purchased from Sigma-Aldrich.

Cell cultures and lithium carbonate (Li_2CO_3) treatment

SH-SY5Y human neuroblastoma cells, a thrice-cloned subline of bone marrow biopsy-derived line SK-N-SH (ECACC #86012802) (generous gift from Cécile Monnet, Centre de Physiopathologie de Toulouse-Purpan, Inserm U563, Toulouse, France) were cultured in Dulbecco's modified Eagle Medium (DMEM, 25 mM glucose) supplemented with 10 % fetal calf serum (FCS) and 20 µg/mL of gentamycin and grown at 37 °C in humidified 5 % CO_2 atmosphere. Cells were seeded at a density of 500,000 cells per T25 flasks containing 8 ml culture medium and allowed to attach overnight. 0.5 mM Li_2CO_3 was added 18 h following seeding in treated cells line (Li^+). Each week, cells from control (C) and experimental cultures were dissociated by trypsin–EDTA treatment, counted and then re-seeded at the same density for more than 10 months. Control (C) and treated (Li^+) cells were periodically collected for analysis.

Analysis of GSK3 and β-catenin protein expressions on Western blot

Cell layers were rinsed with ice-cold phosphate buffer saline (PBS), collected into a lysis buffer (PBS containing 0.5 % NP-40 and protease inhibitors: 10 mM EDTA, 2 mM PMSF, 5 mM NEM, 1 µg/mL aprotinin, pH 7.4), and stored at −20 °C until use. 100 µl of lysis buffer was used for about 10^6 cells. Cell lysates were sonicated for 10 s and protein concentration was determined using the Lowry's method [25]. Equal amounts of protein (20 µg) from each homogenate were separated by SDS PAGE according to Laemmli's method [26] and electroblotted (100 V, 75 min) onto 0.45-µm-pore nitrocellulose membranes.

To saturate the unspecific sites, the membranes were incubated for 1 h at 37 °C into a Tris-buffered saline (TBS: 10 mM Tris, 140 mM NaCl, pH 7.4) containing 0.1 % Tween 20 (TBST). The membranes were then incubated for 1 h at room temperature under continuous shaking in the presence of the mouse antibodies against GSK3 αβ, phospho- GSK3 αβ, β-catenin and phospho-NF-H (RNF405) diluted, respectively, to 1/40,000, 1/1,000, 1/20,000 and 1/1,000 in TBST and allowed to stand overnight at 4 °C. After washing into (1) TBS for 5 min, (2) TBS containing 0.1 % Nonidet-P40 for 15 min, and (3) TBS for 5 min (twice), the blots were incubated at room temperature, in darkness, for 2 h under continuous shaking in the presence of a peroxidase-conjugated secondary antibody diluted in TBS containing 3 % dried skimmed milk. After washing, membranes were incubated with the chemo luminescent substrate for 2 min at room temperature and then exposed to Bio Max light-1 film (KODAK).

Indirect immunofluorescence of cultured cells

Cells from control (C) or treated (Li) with lithium (28 weeks) were seeded in glass coverslips put in 24

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wells culture plates at a concentration of 50,000 cells/wel/800 µl. 48 h later, cells were fixed in methanol for 5–10 min at −20 °C, Permeabilized with acetone for 2 min and then were stored at −20 °C for until use. For staining, cells were rehydrated in PBS for 10 min and incubated for 2 h, in humidified atmosphere, with Primary antibodies, i.e., anti-α-tubilin, anti-phospho-NF-H and anti-β-catenin, diluted to 1/100, 1/50 and 1/200, respectively. After incubation in primary, cells were washed in PBS 3 times for 5 min each wash and then incubated for 2 h, in humidified atmosphere, with secondary antibodies (Anti mouse-Ig-G) coupled to FITC fluorochrome diluted to 1/200 in PBS. After that, cells were rinsed 4 times for 5 min each one and coverslips were mounted by fluoprep on glass slides. Cells side was placed down on drop of mounting media on glass slide. Coverslips were allowed to dry overnight before viewing. Cells were viewed under UV light using a fluorescence microscope Olympus BH-2. Photographs were taken using OM4 Olympus machine and ektachrome 320 T films.

Gene expression analysis using human neurobiology arrays

The culture medium of control cells or those cultured for 42 weeks in presence of 0.5 mM of lithium carbonate was rapidly sucked out and cell layers were frozen at −80 °C until analysis.

Total RNAs were extracted using the RNAble® procedure (Eurobio). cDNA probe synthesis and hybridization were carried out as specified by the manufacturer (Clontech). Briefly, the polyA$^+$ RNA fraction was isolated with biotinylated oligo-dT bound to streptavidin-coated magnetic beads. ^{32}P-labeled cDNA probes were synthesized on captured polyA$^+$ RNA with a MMLV reverse transcriptase in the presence of [α-^{32}P] ATP and a gene specific primer mix. This mix only contained primers for genes represented on the array. This procedure provides the lowest background signal by concentrating the entire radioactive label into the newly synthesized DNA. ^{32}P-labeled cDNA probes were purified on a chromatography column and labeling was checked by scintillation counting. Probes were then hybridized onto the cDNA arrays which contain duplicate spots of cDNA fragments from 588 human genes involved in the neurobiology. Hybridized membranes were imaged with a scanner phosphorimager. Comparison of expression profiles and quantitative analysis was carried out using the Atlas image™ (Clontech) program. The global normalization mode was used to normalize the signal intensity between the two arrays being compared.

Results

Effect of lithium on morphological cells changes

Hemalun eosin staining assay was used to evaluate morphological changes of SH-SY5Y cells under long-term lithium treatment at therapeutic relevant concentration (Fig. 1). Microscopic observation of stained cells showed that the number of cells at mitosis and therefore the cell density were higher in Li cultures than in C cultures. Moreover, lithium treatment has induced neurite outgrowth and neurite branching leading to the establishment of neuronal networks. In C cultures, axons seem to be more long and thin than those of Li cultures where lithium induces axonal spreading along the entire axon shaft and reduces axon length.

Effect of lithium on cytoskeleton changes

Analysis of microtubules organization

Immunofluorescence staining of α-tubulin polymers showed that microtubules in the soma or in the cell ramifications have the same staining level in C and Li cultures (Fig. 2). The Li cells tend to form cellular aggregates which explain the difficulty of the enzymatic dissociation of the cells layer in Li cultures compared to C. This aggregation could be related to the lithium effect on cells adhesion molecules. Besides, Fig. 2 showed that Li cells have more of neurites, growth cones and synapses. Moreover, neurites were shorter and thicker in Li than in C cells.

Analysis of neurofilaments organization

NFs were immunodetected with an anti-NF-H antibody. The revealed zones are the soma and the neurites. The immunostaining was more intense in the perikaryon and growth cones of Li cells compared to controls (Fig. 3b). Furthermore, western blot assay showed that phosphorylation level of NFs was decreased in lithium treated cells (Fig. 3a).

Effect of lithium on β-catenin expression

In attempt to understand the cause of dissociation difficulty of Li cells, we have used the anti-β-catenin antibody to visualize intercellular junction zones. As shown in Fig. 4, we have observed a microgranular staining in the soma plasmic membrane and in the presynaptic terminals of lithium treated cells. Whereas, this staining has seemed absent in C cells.

Differential expression of genes involved in the cytoskeleton modulation using cDNA macroarrays:

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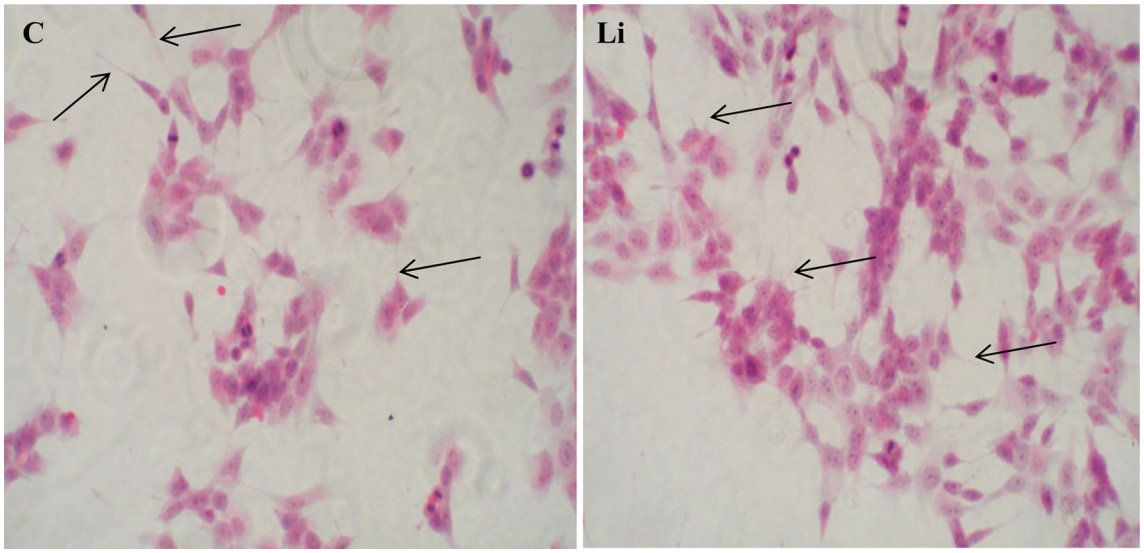


Fig. 1 Effect of lithium on morphological appearance of SH-SY5Y cells: photographs of cultures were taken 2 days after the seeding of control SH-SY5Y cells (C) or previously cultured for 52 weeks in presence of 0.5 mM lithium carbonate (Li). Haemalun-eosin staining; magnification: ×200. → Neurites: long and thin in C cells, short and thick in Li cells

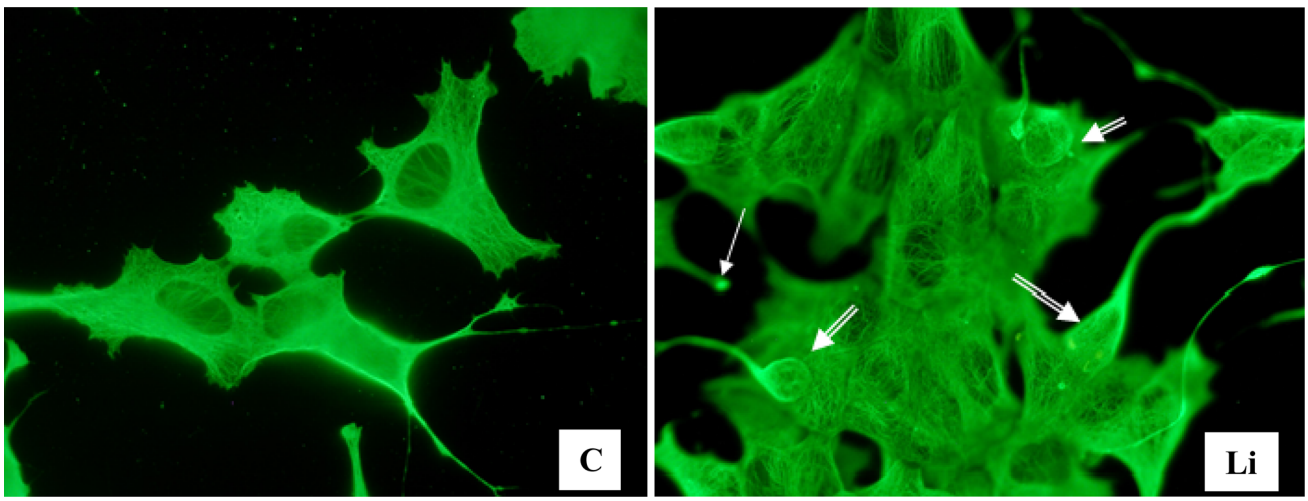


Fig. 2 FITC Immunofluorescence staining of α-tubulin in SH-SY5Y previously cultured in the absence (C) or presence of 0.5 mM Li_2CO_3 (Li) for 28 weeks. Magnification: x 600. → Growth cone. ⟹ Synapse

Expression of genes was analyzed in cells cultured for 42 weeks in presence of therapeutic lithium concentration (0.5 mM). Ratio between expression of transcripts from Li treated cells and controls is reported in Table 1. As recommended by the manufacturer, changes were only considered as significant when the ratios were <0.5 or >2.

Results showed that the transcription levels of genes coding for CK2 (ratio 2.7) and thymosin beta-10 (ratio 2) were significantly increased in (Li) cells compared to (C). Inversely, gene coding for RACK1 (ratio 0.51) seemed to be decreased.

Effects of lithium on glycogen synthase kinase 3 (GSK3)

Effect of lithium on GSK3 expression

As shown in Fig. 5, long-term exposure to lithium (0.5 mM) did not induce significant changes in expression of both total form or phosphorylated form (on serine 21 for GSK3α or on serine 9 for GSK3β) of GSK3 in Li cells compared to that in control cells.

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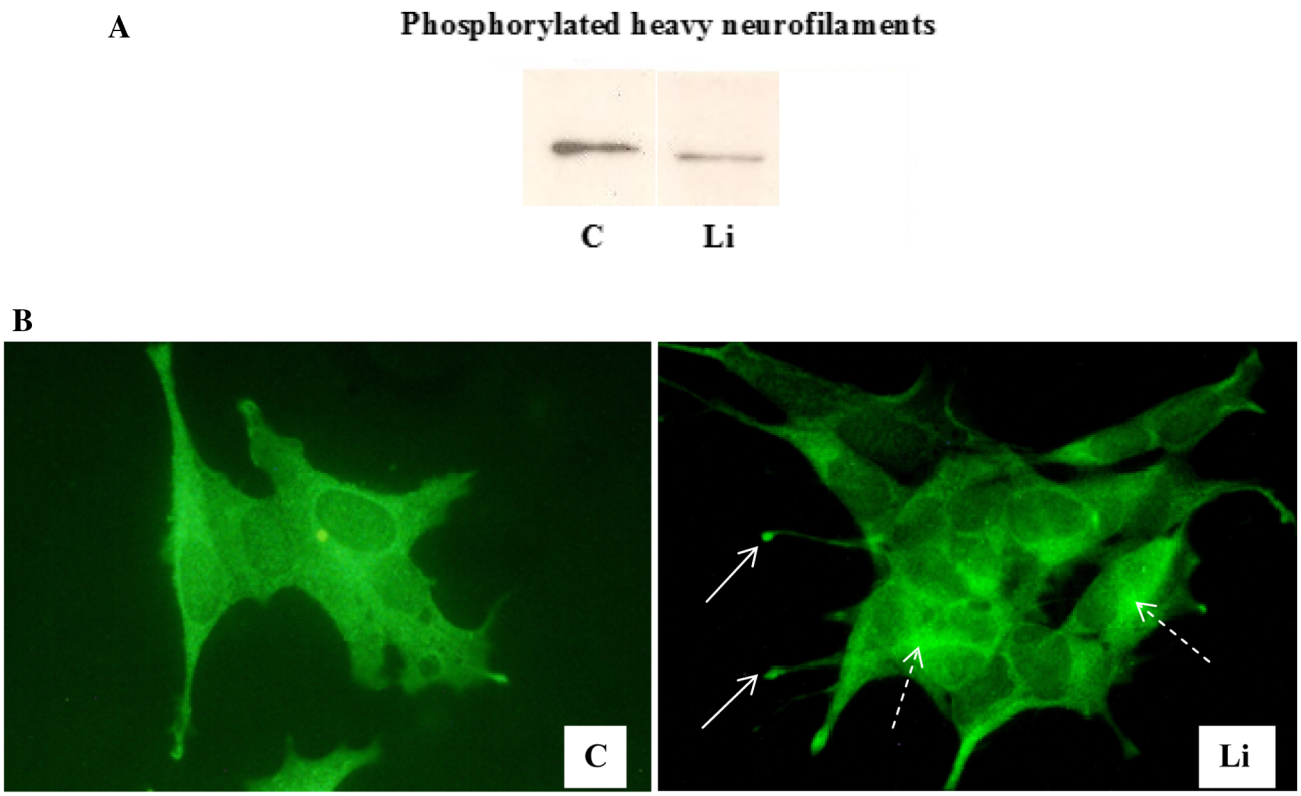


Fig. 3 **a** Immunoblot showing p-NF-H expression in NP40 extracts of SH-SY5Y cells from control (C) or lithium treated cells (Li). Equal amounts of protein (20 µg) were resolved by SDS-PAGE on 10 % polyacrylamide gels. **b** FITC Immunofluorescence staining of p-NF-H in SH-SY5Y previously cultured in the absence (C) or presence of 0.5 mM Li_2CO_3 (Li) for 28 weeks. Magnification: ×1,000. → Growth cone. - - - - - -> Perinuclear zone

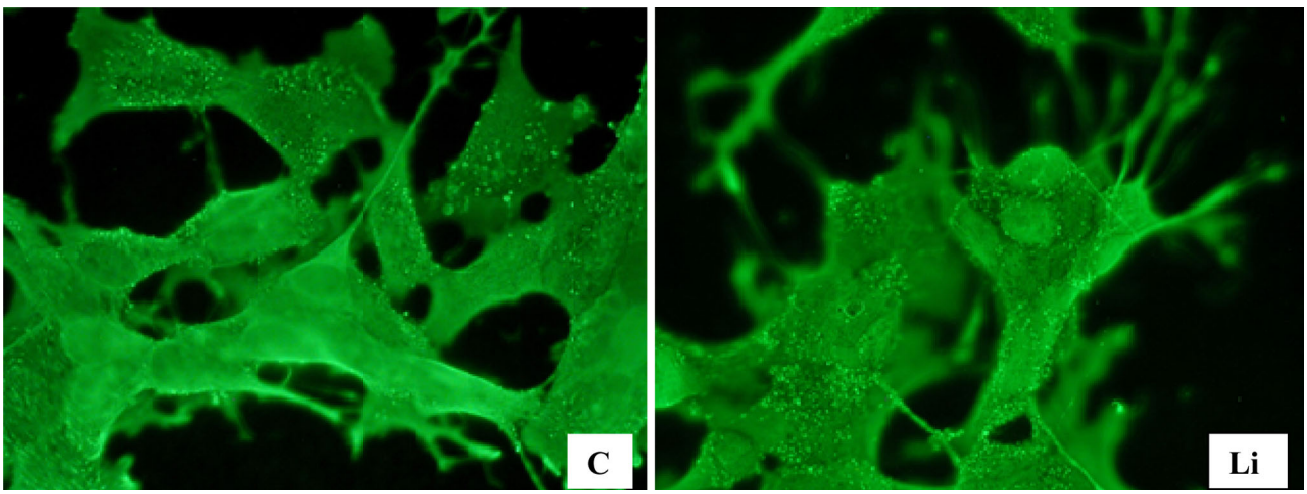


Fig. 4 FITC Immunofluorescence staining of β-catenin in SH-SY5Y previously cultured in the absence (C) or presence of 0.5 mM Li_2CO_3 (Li) for 28 weeks. Magnification: ×600

Effect of lithium on GSK3 activity (expression level of phosphorylated β-catenin)

Evaluation of the effect of lithium on GSK3 activity was performed by highlighting the expression level of phosphorylated form of β-catenin protein. The soluble form of this protein was extracted in NP40 buffer, whereas, its cytoskeleton associated form was extracted in PBS buffer containing urea. The two forms were quantified with antibodies witch recognized phosphorylated forms in serine

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Table 1 Expression level changes of transcripts from genes involved in cytoskeleton modulation of SH-SY5Y cells cultured for 42 weeks in presence of 0.5 mM Li_2CO_3

Gene/protein classification	Gene/protein name	Ratio
Actin G sequestration	Thymosin β-10	2
Cell signaling and intracellular kinase network members	casein kinase II (CK2)	2.7
Signal transduction	Receptor for Activated C Kinase (RACK1)	0.51

Values correspond to the mean of the ratios of two spots from one experiment

31 and 37. The Fig. 6 demonstrated that the expression level of β-catenin (92–97 kDa) was higher in urea extract than in NP40 extract. However, the phosphorylation levels were comparable between controls and Li (28 and 53 w) cells in NP40 and urea extracts. Similar results were obtained for the β-catenin cleavage products (45–55 kDa). The stability of β-catenin phosphorylation level proves that GSK3 activity was not modified by this treatment.

Discussion

The neuronal cytoskeleton is much more than a structural framework, but is a dynamic system contributing to neural plasticity [27]. In fact, neuronal differentiation is accompanied by profound morphological alterations, including the elaboration of dendritic and axonal neurites. The relative requirement for plasticity in outgrowing neurites versus stabilized axons is reflected in their cytoskeletal composition [28].

Lithium in addition to being neuroprotective is known to promote new neurite sprouting and enhance neurite outgrowth in vitro models [29]. Indeed, Garcia-Perez et al. [30] first showed that lithium induced neurite outgrowth in Neuro-2a cells. They used an assay that measured the proportion of cells with neurites, rather than the length of neurites. Furthermore, Muñoz-Montaño et al. [31] also showed that high levels of Li inhibit neurite outgrowth in cerebellar granule cells, but lower levels (1–5 mM) promote it.

In this work, we investigated the effect of lithium treatment on cytoskeleton proteins changes inducing neuritogenesis. We also studied the involvement of GSK3 protein in lithium action. SH-SY5Y microscopic observation and IIF immunostaining of tubulin and neurofilaments showed that chronic lithium treatment at therapeutic relevant concentration induced neurite outgrowth and neurite branching leading to the establishment of neuronal networks. Moreover, neurites were shorter and thicker in Li than in C cells. Therefore, Li cells have more of neurites, growth cones and synapses than C cells. Same observation was made by Williams et al. [32]; lithium treatment at a therapeutically relevant concentration induced axonal branching and caused spreading of growth cones. In this context, Fiona et al. [33] suggested that the morphological changes induced by lithium are dose dependent. While low concentrations of lithium increase growth cone size, high lithium concentrations induce axonal spreading along the entire axon shaft and reduce axon length.

Our results showed that gene coding for thymosin β-10 was significantly increased by lithium under our condition. Thymosin β-4 and thymosin β l0, which are the most abundant β-thymosins, were identified as a major actin monomer-binding protein in platelets. Both β-thymosins bound actin monomers and decreased the steady state actin polymer concentration [34]. In NIH 3T3 cells, the increase in the concentration of the G-actin-sequestering peptide thymosin β 4 cause an increase of the G-actin sequestered levels. These cells coordinately increase several cytoskeletal and adhesion plaque proteins [35]. In our case, the increase of thymosin β-10, which causes the increase of G-actin sequestration and the inhibition of its polymerization, could explain the reduction of neurite length. Besides, it promotes growth cones plasticity and plaque adhesion establishment.

Expression level of gene coding for CK2 was also increased. CK2 is a serine/threonine protein kinase with prominent prosurvival functions. It is markedly augmented in a wide range of cancers and appears to enhance tumor

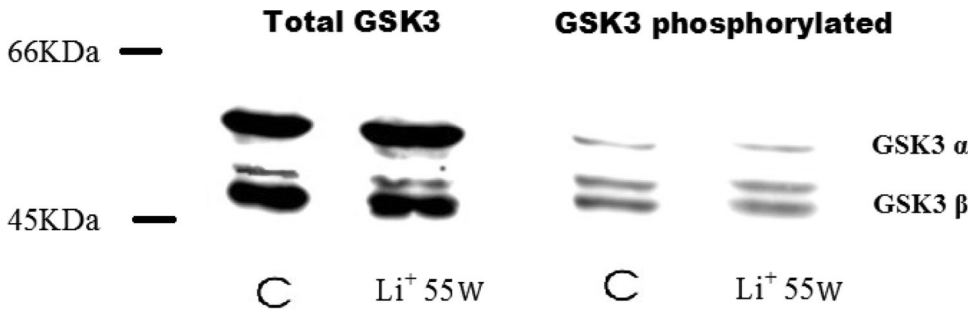


Fig. 5 Immunoblot showing the expression of total GSK3 and of GSK3 phosphorylated on serine 21 for GSK3α or serine 9 for GSK3β in SH-SY5Y controls cells (C) or cells cultured in presence of 0.5 mM lithium for 55 weeks (Li 55 w)

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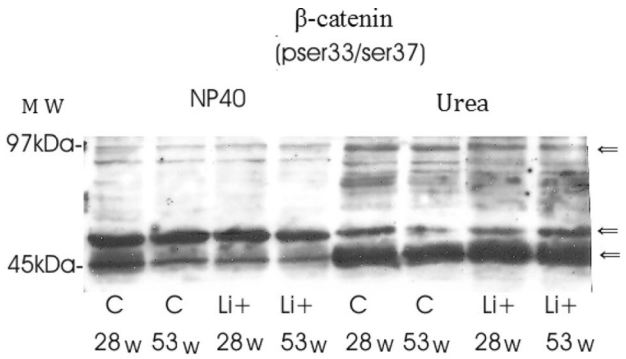


Fig. 6 Immunoblot showing the expression of β-catenin phosphorylated on serine 33 and serine 37 in NP40 and urea extracts of SH-SY5Y cells from control line (C) or cultured in presence of 0.5 mM lithium for 28 (Li 28 w) or 53 weeks (Li 53 w)

growth and angiogenesis [36]. It is implicated in several cellular pathways controlling proliferation and differentiation. CK2 also inhibits apoptosis and stimulates Wnt signaling pathway [37]. It was demonstrated that depletion of casein kinase 2 by antisense oligonucleotides blocks neuritogenesis and the ability of MAP-1B to bind to microtubules [38]. Hence, by increasing CK2 level, lithium enhances neuritogenesis and microtubule stability.

Conversely to thymosin β- 10 and CK2, gene coding for RACK1 was inhibited by lithium treatment. In mammals, RACK1 regulates several processes that involve contact with the extracellular matrix, such as cell spreading, the establishment of focal adhesions and cell–cell contacts. RACK1 interacts particularly with two important focal-adhesion components: talin and vinculin, cytoskeletal proteins which play a key role in integrin-mediated cell adhesion and migration [39].

To visualize intercellular junction zones, we have used the anti-β-catenin antibody. β-Catenin is an 88-kDa member of the armadillo family of proteins that is associated with the cadherin-catenin complex in the plasma membrane. This complex interacts dynamically with the actin cytoskeleton to stabilize adherens junctions [40]. Binding of β-catenin to the actin cytoskeleton through cadherins could lead to changes in the actin cytoskeleton resulting in axonal spreading [33]. We have observed that in Li cultures, this protein was localized in the soma plasmic membrane and in the presynaptic terminals. Brembeck et al. [41] reported that β-catenin controls E-cadherin-mediated cell adhesion at the plasma membrane and mediates the interplay of adherens junction molecules with the actin cytoskeleton. This could explain dissociation difficulty of cell aggregates of lithium-treated cultures.

The most extensively studied signaling pathway associated with lithium's neuroprotective action involves the inactivation of glycogen synthase kinase-3β (GSK3 β), a proapoptotic enzyme responsible for tau hyperphosphorylation in Alzheimer disease [42].

In an attempt to elucidate molecular mechanism by which lithium regulates cytoskeletal protein function, and the consequential effects on neuronal architecture, we have examined the GSK3 expression and activity. Our results presented in GSK3 and β-catenin immunoblots indicate that lithium acts without modifying GSK3 expression and/or activity. So, in our experimental conditions, the mechanism by which lithium performed its action is independent of GSK3 β inhibition pathway. This indicates that lithium could act on pathways that are independant of GSK3β. We have found similar results in vivo. In fact, 0.8 mM lithium was able to enhance neurogenesis in mice hippocampus and enthorinal cortex after 3 months of treatment. This was highlighted by the increase of axon diameters and the enhancement of neurites growth in CA3 area of hippocampus. Furthermore, neuron network was enriched in enthorinal cortex. This enrichment was proved by the increase of myelination density. The treatment was without effect on both GSK-3αβ and phosphorylated β-catenin expression levels in brains of treated mice. This suggests that lithium has not modified GSK-3 expression and activity under this treatment [6]. More recently, we have demonstrated that chronic exposure (25–50 weeks) to low lithium concentrations ((0.5 mM Li_2CO_3) induces adaptive changes in metabolism of SH-SY5Y cells involving a higher cell growth rate and a better resistance to oxidative stress. This effect was mediated by pyruvate accumulation in extracellular medium, this molecule having an antioxidant and neuroprotective proprieties [43]. In an other work, we have investigated the molecular mechanism underlying the neuroprotective effect of lithium on SH-SY5Y cells following chronic treatment at therapeutically relevant concentration. We have proved that it could be mediated by its ability (lithium) to regulate various phosphatases and kinases activities. This acts on post-translational level and then on protein activities [44].

It has been shown that lithium through initial actions on GSK-3, potentiate GSK-3 serine phosphorylation, resulting in synergistic neuroprotective effects [45]. GSK-3 inhibition, elicited by enhanced GSK-3 serine phosphorylation or other mechanisms, is expected to activate or inhibit an array of transcription factors, causing upregulation of neuroprotective and/or neurotrophic proteins but down-regulation of pro-apoptotic proteins [46].

Garcia-Perez et al. [30] first showed that lithium induced neurite outgrowth in Neuro-2a cells. Neuritogenesis occurs due to GSK-3 inhibition. Thus Neuro-2a cells extend neurites when GSK-3 kinase activity is inhibited, suggesting that GSK-3 activity is inhibitory to neurite outgrowth. However, GSK-3 activity appears to be important

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for neurite outgrowth in other systems [47]. Therefore, it seems that there is an optimal level of GSK-3 activity that promotes neurite outgrowth. This may reflect the need for a particular level of phosphorylation of substrates; if this is the case, the basal GSK-3 activity and the amount of phosphatase activity indifferent cell types will dictate the effects of Li in each case [47]. Indeed, lithium modulates signals impacting on the cytoskeleton at multiple levels, including signals from GSK-3β, cyclic AMP dependent kinase and protein kinase C. These actions may be critical for the neural plasticity involved in mood recovery and stabilization [27].

Conclusion

We concluded that long-term lithium treatment, at therapeutic dose, induced cytoskeleton protein changes to promote neuritogenesis in SH-SY5Y. The mechanism underlying lithium action was not related to the inhibition of GSK3 activity.

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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